Protectin DX, a double lipoxygenase product of DHA, inhibits both ROS production in human neutrophils and cyclooxygenase activities.

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Miao Liu[1*] · Tarek Boussetta[2*] · Karama Makni-Maalej[2*] · Michèle Fay[2] · Fathi Driss[2] · Jamel El-Benna[2] · Michel Lagarde[1] · Michel Guichardant[1]**

[1]Université de Lyon, UMR 1060 Inserm (CarMeN), IMBL/INSA-Lyon, Villeurbanne, F69621, France

[2]Inserm, UMR 773, CRB3, Université Paris 7 Denis Diderot, Faculté de Médecine, site Bichat, Paris, F75018, France

[*]contributed equally to this work

[**]**Corresponding author:**

Michel Guichardant

Tel: 33(0)472438215, Fax: 33(0)472438524,

E-mail: michel.guichardant@insa-lyon.fr

Abbreviations

ARA, arachidonic acid; BSTFA, *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide; CLA, conjugated linoleic acid; ClnA, conjugated linolenic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; ECL, enhanced chemiluminescence; EPA, eicosapentaenoic acid; 5-HETE, 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; fMLF, formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte macrophage-colony stimulating factor; HBSS, Hank's balanced salt solution; HOCl, hypochloric acid; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; IL, interleukin; LA, linoleic acid; LPS, lipopolysaccharides; LTB_4, leukotriene B_4; MPO, myeloperoxidase; NOX, NADPH oxidase; PAF, platelet-activating factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDX, protectin DX; PG, prostaglandin; phox, phagocyte oxidase; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; RP-HPLC, reverse phase high performance liquid chromatography; sLOX, soybean lipoxygenase; TLC, thin-layer chromatography; TMB, tetramethyl benzidine; TNBS, trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor-α.

Abstract

Neutrophils play a major role in inflammation by releasing large amounts of reactive oxygen species (ROS) produced by NADPH oxidase (NOX) and myeloperoxidase (MPO). This ROS overproduction is mediated by phosphorylation of the NOX subunits with an uncontrolled manner. Therefore, targeting neutrophil subunits would represent a promising strategy to moderate NOX activity, lower ROS, and other inflammatory agents, such as cytokines and leukotrienes, produced by neutrophils. For this purpose, we investigated the effects of protectin DX (PDX) - a docosahexaenoic acid (DHA) di-hydroxylated product which inhibits blood platelet aggregation - on neutrophil activation *in vitro*. We found that PDX decreases ROS production, inhibits NOX activation and MPO release from neutrophils. We also confirm, that PDX is an anti-aggregatory and anti-inflammatory agent by inhibiting both cyclooxygenase-1 and -2 (COX-1 and COX-2, E.C. 1.14.99.1) as well as COX-2 in lipopolysaccharides (LPS)-treated human neutrophils. However, PDX has no effect on the 5-lipoxygenase pathway that produces the chemotactic agent leukotriene B_4 (LTB_4). Taken together, our results suggest that PDX could be a protective agent against neutrophil invasion in chronic inflammatory diseases.

Keywords

Human neutrophils; inflammation; protectin DX; NADPH oxidase; ROS-derived oxidative stress

Introduction

Chronic inflammation is involved in various diseases including atherosclerosis [1], bowel disease [2, 3], cancer [4], cardiovascular diseases [5], the metabolic syndrome [6], and rheumatoid arthritis [7]. They all have in common [8] excessive production of pro-inflammatory mediators such as interleukin (IL)-1, IL-6, IL-8 [9], granulocyte macrophage-colony stimulating factor (GM-CSF) [10], leukotriene B_4 (LTB_4), platelet-activating factor (PAF), and tumor necrosis factor-α (TNF-α) [8]. Highly activated inflammatory cells such as neutrophils, monocytes and macrophages [11], and production of reactive oxygen species (ROS) [12–15] are common features. A major source of ROS at inflammation sites is the reduction of an oxygen molecule to superoxide anion ($O_2^{-\cdot}$) by neutrophil enzyme complex NADPH oxidase (NOX) [16], and hypochloric acid (HOCl) produced by myeloperoxidase (MPO) from hydrogen peroxide [13]. In physiological situations, NOX activation in neutrophils is tightly regulated to avoid tissue damage. However, a dysregulation occurs in chronic inflammatory states where NOX subunits are strongly phosphorylated and translocated to cell plasma membrane, resulting in highly active oxidase overproducing ROS [17]. Targeting NOX subunits to decrease ROS overproduction would then represent a strategy to develop new anti-inflammatory approaches. Dietary manipulation is a possible way to prevent inflammatory diseases that involve polymorphonuclear neutrophil (PMN) activation and oxidative stress. As a matter of fact, dietary long-chain n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, present in marine food, have been widely used. As an example, they have been shown to attenuate inflammation and neutrophil infiltration in an experimental model of colitis [18]. N-3 PUFA may be converted into active oxygenated derivatives named protectins and resolvins which may fight inflammation [19]. Moreover, conjugated double bond-containing PUFA, like conjugated linoleic (CLA) and linolenic (ClnA) acids have been reported to decrease the incidence of tumors in mice, to inhibit cell proliferation and invasion of cancer cells by promoting apoptosis [20–22]. We have previously found that punicic acid, a conjugated triene PUFA found in high percentage (70%) in pomegranate seed oil, exerts a potent anti-inflammatory effect through inhibition of TNFα-induced priming of neutrophil NOX, by targeting the p38MAPK/Ser^{345}-$p47^{phox}$ axis, and by decreasing neutrophil degranulation and MPO release [23]. On the other hand, oral administration of punicic acid or pomegranate seed oil to rats inhibits trinitrobenzene sulfonic acid (TNBS)-induced colitis and decreases ROS-induced tissue damage through a down regulation of neutrophil activation [23].

Recently, our group identified new di-oxygenated derivatives from PUFA, called poxytrins, which inhibit blood platelet aggregation [24]. Among them, protectin DX (PDX) deriving from DHA [25] shares some structure similarity with other conjugated triene fatty acids, like punicic acid and oleostearic acid. PDX is an isomer of protectin/neuroprotectin D1, which is known to have anti-inflammatory properties [19, 26]. More recently, PDX (called PD1 isomer, even PD1 through the paper) has been shown to inhibit the influenza virus replication [27], which makes it a relevant molecule to fight this infectious disease. The aim of the present study was to test whether PDX may have anti-inflammatory effect by modulating neutrophil activation. A second goal was to precise the anti-inflammatory potential of PDX through cyclooxygenase inhibition. We found that PDX decreases ROS produced by neutrophils stimulated with formyl-methionyl-leucyl-phenylalanine (fMLF), and phorbol myristate acetate (PMA) *in vitro*, and partly inhibited neutrophil degranulation and MPO release by fMLF-triggered neutrophils. Also, PDX is an inhibitor of COX-1, as well as COX-2 which the activity is enhanced during inflammation, but fails to inhibit the 5-lipoxygenase pathway. Altogether these effects provide evidence that PDX may be an anti-inflammatory agent acting through original mechanisms.

Materials and Methods

Reagents and antibodies

PMA, fMLF, protease and phosphatase inhibitors, DHA, arachidonic acid (ARA), soybean lipoxygenase (sLOX) (E.C. 1.13.11.12, Type 1-B), COX-1 from sheep, and human recombinant COX-2 (E.C. 1.14.99.1), *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were from Sigma-Aldrich (St Quentin Fallavier, France). Endotoxin-free buffers and salt solutions were from Invitrogen/Life Technologies SAS (St Aubin, France). Anti phospho-Ser^{345}-$p47^{phox}$ and anti-$p47^{phox}$ were from Cell Signaling/Ozyme (St Quentin Yvelines, France). Anti MPO antibody was from Abcam. Organic solvents were from Carlo-Erba. All chemicals used were reagent grade or with the highest quality available. [^{3}H]-ARA (6.6×10^9 Bq/mM) was from Perkin Elmer, USA.

PDX synthesis

PDX was prepared as previously described [25]. Briefly, 200 µM DHA was incubated with soybean lipoxygenase in sodium-borate buffer (pH 9.0) on ice for 30 min, synthesized hydroperoxides were then reduced by $NaBH_4$ for 15 min, and the reduction step was stopped by adding acetic acid to pH 3.0. PDX was extracted by using a C18-Sep Pak cartridge, collected by reverse phase high performance liquid chromatography (RP-HPLC), and measured according to its UV absorbance.

Preparation of human neutrophils and measurement of ROS production

Human neutrophils were obtained from healthy donors, in using LPS-free conditions by one-step purification on polymorphprep dextran sedimentation as previously described [28]. Briefly, freshly drawn blood was added to cold dextran solution (0.1 mM dextran T500, 40 mM EDTA, 120 mM NaCl, pH 7.4) in a screw-cap polypropylene tubes, and mixed gently. After at least 1 hour on melting ice, the upper solution was collected and mixed with phosphate-buffered saline (PBS) pH 7.4. Cold Ficoll (density 1.077) was added carefully to the bottom of the tubes and were further centrifuged at 500 g for 20 min at 4°C. After centrifugation, lymphocytes were removed and the pellets were re-suspended in cold PBS. Red cells were lysed by hypotonic shock. Neutrophils were then washed twice with PBS and kept on ice until analysis.

ROS production was measured with a luminol-amplified chemiluminescence method: human neutrophils were suspended in Hank's balanced salt solution (HBSS) containing 10 µM luminol, and pre-heated at 37°C in the thermostated chamber of the luminometer (Berthold-Biolumat LB937) prior to treatment with PDX. Neutrophils were then stimulated with PMA (0.162 mM) or 0.1 µM fMLF, for chemiluminescence recording.

Measurement of released MPO from neutrophils

The release of MPO was performed by measuring its activity in using the H_2O_2-dependent tetramethyl benzidine (TMB)-oxidation assay at 650 nm. Human neutrophils were suspended in HBSS and pre-heated at 37 °C. The cells were treated with PDX, and then stimulated or not with 0.1 µM fMLF. Neutrophils were then centrifuged and MPO activity was measured in supernatants. Total MPO activity has been expressed in mU/min relative to a standard curve established with known amounts of triton X-100-treated neutrophils.

Immunoblotting of MPO released by fMLF stimulated neutrophils was done with primary rabbit antibodies against MPO (dilution: 1/10,000), and with a horseradish peroxidase (HRP)-labeled-goat anti-rabbit antibody (dilution: 1/10,000). The reaction was measured by using enhanced chemiluminescence (ECL) reagents.

Detection of Ser^{345} phosphorylation in neutrophils

Neutrophils were incubated with different concentrations of PDX for 20 min, then treated with 0.1 µM fMLF for 20 min. Cell proteins were denatured in Laemmli buffer [29]. The samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotting was done with primary rabbit antibodies against phospho-Ser^{345} (dilution: 1/10,000) or against $p47^{phox}$ (dilution: 1/5,000), then with HRP-labeled-goat anti-rabbit antibody (dilution: 1/10,000). The reaction was measured by using ECL reagents.

Measurement of 5-lipoxygenase activity in neutrophils

Human neutrophils, suspended in a Tyrode-HEPES buffer containing 2 mM of Ca^{2+}, were pre-incubated with 1µM PDX for 3 min at 37°C, and were then triggered with 1 µM of ionophore A23187 plus 10 µM of ARA for 10 min. The reaction was terminated by acidifying the media to pH 3.0 with acetic acid. Oxygenated metabolites (5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE), LTB_4 and its isomers) were then extracted by solid phase extraction on Sep-Pak C18-cartridges. They were analyzed by RP-HPLC on a Waters XBridge C18 column (4.6×250 mm, 3.5 µm) by using a linear solvent gradient (1 mL/min): solvent A was acetonitrile/water (10:90, v/v) pH 3.0, and solvent B was acetonitrile. Mono-hydroxylated conjugated diene and di-hydroxylated conjugated triene fatty acids were detected with a diode array detector at 235 nm and 270 nm, respectively.

GC-MS measurement of COX-1 and COX-2 activities

Commercial COX-1 and COX-2 were pre-incubated for 15 min in a 0.1 M Tris HCl buffer pH 8.0 with or without 0.5 or 5 µM of PDX, and incubated with 10 µM ARA as a substrate for 10 min. Albumin (45 g/L) was added to enhance the formation of prostaglandin (PG) D_2 and E_2 from PGH_2 at the end of incubation [30]. Reaction was stopped by acidification to pH 3.0 with acetic acid. PGD_2 and E_2 were then extracted by 10 volumes of diethyl ether. Deuterated

PGD_2 and E_2 were used as internal standards for their quantification. Prostaglandins were derivatized into methoxime, pentafluorobenzyl ester and trimethysilyl ether, and further analyzed by NICI GC-MS. Selected ions corresponding to $[M-181]^-$ (loss of the PFB group): m/z: 524 for both derivatized PGD_2 and E_2 and m/z: 528 for their corresponding deuterated internal standards, were used for their measurement.

Measurement of LPS-induced COX activities in human neutrophils

Human neutrophils were treated by LPS (100 ng/mL) in order to overexpress COX-2. After 4 hours, cells were pre-incubated in presence or absence of 10 µM PDX for 3 min at 37°C, and 10 µM radiolabelled ARA (333 Bq/nmol) was added as a substrate for 10 min. After acidification to pH 3.0 with acetic acid, lipids were extracted by 10 volumes of diethyl ether and separated by thin-layer chromatography (TLC). Radioactivity of extracted gel spots corresponding to PGD_2 and E_2 were measured by scintillation counting.

PDX incorporation into neutrophils

10 µM PDX was incubated with isolated neutrophils for 3 min at 37°C. Cells were washed twice with buffer containing 45 g/L fatty acid free albumin. After centrifugation at 500 g for 20 min at 4°C, pellets were extracted using methanol/acidified water and PDX was measured by LC-MS/MS (AB Sciex, Qtrap 4500), using the double 15-lipoxygenase product of arachidonic acid (8,15-diHETE) as an internal standard. Quantification was done using multiple reaction monitoring mode (MRM). Parent ions were m/z: 359 and m/z: 335 and daughter ions m/z 153 and m/z 235 for PDX and 8,15-diHETE respectively.

Statistical analysis

All results are expressed as means ± standard error of the mean (SEM) or means ± standard deviation (SD). One away ANOVA followed by Fisher's protected least significant difference (PLSD) post hoc test was used to assess differences between groups. *P*-value of 0.05 was assumed to represent the level of significance. Statistical significance was determined by using 2-tailed Student's t tests in case of comparison between two groups of values.

Results

PDX inhibits fMLF and PMA-induced superoxide anion from neutrophils

PDX was tested on ROS production in response to PMA. In presence of PDX for 20 min, PMA-induced neutophil production of ROS was decreased. Our results (Fig. 1) show that in absence of PDX, PMA induced a marked ROS production compared to baseline (Fig. 1A), and incubation of neutrophils with PDX prior to the addition of PMA, induced a significant decrease in ROS production (Fig. 1B), indicating that PDX inhibited NOX activity, and/or scavenged the produced ROS. The effect of PDX was dose-dependent from 2.5 µM to 10 µM. At these concentrations, PDX did not affect PMN viability as determined by trypan blue exclusion test (not shown).

We then analyzed the effect of PDX on the neutrophil respiratory burst induced by 0.1 µM fMLF. Figure 2A shows that, similarly to the PMA experiments, the luminescence of neutrophils stimulated with fMLF is increased and lowered by PDX, in a dose-dependent manner, also from 2.5 µM to 10 µM (Fig. 2B). In contrast, PDX had no effect on superoxide production by the *in vitro* xanthine/xanthine oxidase system, and had no effect on H_2O_2 availability as measured by the peroxidase-catalyzed reaction (data not shown). These data suggest that PDX did neither scavenge superoxide anion, nor H_2O_2.

PDX inhibits fMLF-induced MPO release from neutrophils

Neutrophil NOX produces superoxide anion (O_2^-) that is the source of other ROS. O_2^- is spontaneously dismutated into H_2O_2 at acidic pH, within the phagosome or the extracellular medium. The release of MPO catalyzes the conversion of H_2O_2 into the toxic molecule HOCl in presence of chloride ions [31–33]. Also, reaction between H_2O_2 and OCl^- produces singlet oxygen, another reactive oxygen species. Therefore, the activation of NOX synergizes with MPO for inflammatory processes. The effect of PDX was then investigated on neutrophil release of MPO. Human neutrophils were incubated in HBSS medium in presence or absence of PDX for 20 min at 37°C. After washing and centrifugation, PMN were stimulated with 0.1 µM fMLF, and MPO release was assessed by measuring its activity in supernatant of all samples. Figure 3A shows that MPO activity from neutrophils pre-treated with PDX and

stimulated with 0.1 µM fMLF, was significantly decreased in a dose-dependent manner as compared to control (neutrophils treated without PDX, but stimulated with fMLF). To test whether this inhibitory effect may reflect decreased neutrophil degranulation, or is related to decreased enzymatic activity, we performed a western blot analysis of the supernatant from fMLF-stimulated samples, using an antibody directed against human MPO. Results (Fig. 3B) show that the amount of MPO was lower in 5 µM and 10 µM PDX-treated neutrophils than in control (neutrophils treated without PDX, but stimulated with fMLF), whereas we did not find any effect of PDX on the enzymatic activity determined *in vitro* on recombinant MPO activity (data not shown). Taken together our results suggest that PDX exerts an inhibitory effect on stimulated neutrophil oxidative burst and degranulation.

PDX inhibits fMLF-induced $p47^{phox}$ phosphorylation on Ser^{345}

We have previously shown that production of ROS by NOX results from the phosphorylation of its cytosolic subunits, particularly $p47^{phox}$ on Ser^{345}. The effect of PDX on this process is shown in Fig. 4. The level of phosphorylated ^{345}P-Ser on $p47^{phox}$ has been corrected from the amount of total $p47^{phox}$, which shows that PDX significantly lowers the phosphorylation of this protein when induced by fMLF treatment of neutrophils (Fig. 4A and B).

Effect of PDX on COX-1 and COX-2 activities

The inhibition of purified cyclooxygenases (COX-1 and COX-2) by 0.5 µM and 5 µM of PDX were assessed by GC-MS measurement of both PGD_2 and E_2 which represent the global prostaglandin synthesis after albumin addition and acidification that convert PGH_2 into PGD_2 and E_2 (see methods). Results reported in Table 1 show that 0.5 µM and 5 µM PDX significantly decreased prostaglandin production through COX-1 by around 55% and 75%, respectively. Interestingly, the inhibition of COX-2 by PDX seems even more efficient as the decreased prostaglandin production through COX-2 attained 70% and 80% in response to 0.5 µM and 5 µM PDX, respectively. This means that the decreased production of pro-inflammatory prostaglandins by PDX may substantially contribute in its anti-inflammatory effects.

Effect of PDX on prostaglandin production in human neutrophils treated by LPS

Neutrophils were treated by LPS in order to overexpress COX-2. Radiolabelled ARA was then used as a substrate for the neutrophil cyclooxygenase in the presence or absence of 10 µM PDX, in order to exclude the contribution of released endogenous ARA in response to LPS. COX activity was assessed by measuring the radioactive PGD_2 and E_2 as representatives of prostaglandin production. Results (Fig. 5) show that such a production was enhanced by 121% following the LPS treatment, compared to non-stimulated cells, reflecting the expected COX-2 overexpression. 10 µM PDX significantly decreased prostaglandin production by around 25% in LPS-treated neutrophils. The inhibition by PDX was not visible in non-treated neutrophils compared to control (not shown).

Effect of PDX on 5-lipoxygenase activity in activated human isolated PMN

The 5-lipoxygenase pathway is a relevant one to promote inflammation through the ARA metabolite LTB_4. The 5-LOX end-products are LTB_4 and its geometrical and stereochemical isomers (LTB_4 isomers), as well as 5-HETE. Measuring all those end-products provides a global view of the 5-lipoxygenase pathway acting upon ARA. Results summarized in Table 2 show that even at 10 µM, PDX did not inhibit the 5-lipoxygenase pathway, meaning that the inhibition of the cyclooxygenase reported above is rather specific.

PDX incorporation into neutrophils

The IC_{50} for the inhibition of isolated COX enzymes by PDX being much lower (less than 0.5 µM) than in neutrophils (more than 10µM), studies on the uptake of PDX has been done. Incubation of neutrophils with 10 µM PDX led to low incorporation (8.5 +/- 0.7 %) as measured by LC-MS/MS. No degradation products were detected, and the remaining PDX (not incorporated) was measured in the supernatant fraction.

Discussion

The present study shows that PDX inhibits both ROS production and MPO release by human neutrophils. Also, the inhibition of fMLF-induced activation of NOX results from the inhibition of the phosphorylation of p47phox on Ser^{345}.

Several cytokines, including TNF-α, are involved in many inflammatory states. As a matter of fact, anti-TNF-α antibodies are used to treat some severe forms of inflammatory bowel disease [34]. This cytokine primes the neutrophil ROS production that is a key process in inflammation [35]. Primed neutrophils have been reported in diverse inflammatory states [36, 37]. One molecular mechanism involved in this priming process is the phosphorylation of $p47^{phox}$ on Ser^{345}. Anti-inflammatory interventions could use nutritional approaches to decrease such a phosphorylation and subsequently ROS overproduction, without altering that one which is required for innate immunity and host defence. The present study shows that PDX, a double lipoxygenase product of DHA, is able to slow-down NOX activation and subsequent ROS-derived oxidative stress. MPO activity was also decreased by PDX to the same extent. As MPO and ROS (especially H_2O_2) generate toxic molecules at the inflammatory sites, the inhibition by PDX supports the potential action of this DHA metabolite as an anti-inflammatory agent. However, this protective effect needs to be confirmed *in vivo*.

Other fatty acids may affect ROS production in neutrophils. Linoleic acid (LA), the main consumed fatty acid in western countries [38], may act synergistically with TNF-α to affect ROS production [39, 40]. The LA product ARA can directly activate neutrophils [41] through enzymatic conversion into pro-inflammatory oxygenated metabolites such as LTB_4 and PGE_2 [42–44]. By contrast, n-3 PUFA such as EPA and DHA, provided by marine lipids, are oxygenated into less inflammatory and even anti-inflammatory derivatives counteracting the effects of ARA-derived eicosanoids [45]. Indeed, Hardy et al. have shown that pre-treatment with ARA, enhances fMLF- or PMA-stimulated neutrophils, then inducing more superoxide anion production than in non ARA-enriched cells [46]. In contrast, a recent study reported that EPA and DHA could increase the response of neutrophils to TNF-α [47]. None of these *in vitro* and *in vivo* experiments have addressed the molecular mechanism that might explain the increased ROS production.

Nutritional doses of EPA and DHA have been used in humans to prevent inflammatory states, and to lower the level of circulating inflammation markers [45]. The inhibitory effect of PDX on prostaglandin synthesis by either sheep COX-1 or human recombinant COX-2 is

quite relevant in this respect, especially at 0.5 µM. This was also observed to a lesser extent in neutrophils treated by LPS. However, this inhibition is in the same order if we consider that only 8% of PDX was incorporated into the neutrophils after 10 µM incubation. This means that PDX would be more relevant when produced inside neutrophils through their own 15-LOX.

In addition to the COX-1 inhibition, already observed in relation with the inhibition of platelet aggregation [24], PDX appears to be an even more potent inhibitor of COX-2 which is the inducible enzyme, although detectable at low level in most normal tissues. It becomes abundant in activated macrophages and other cells at sites of inflammation. The anti-inflammatory potential of PDX is quite specific since it fails to inhibit the 5-lipoxygenase pathway. Therefore, PDX appears to be an original anti-inflammatory agent to play a protective role in chronic inflammatory diseases.

We conclude that PDX inhibits fMLF-induced ROS production by targeting the neutrophil $p47^{phox}$/NOX axis and MPO release as well. Besides its inhibitory effects on platelet aggregation through inhibition of COX-1, that is confirmed against purified COX-1, PDX inhibits COX-2 and then could protect against inflammation. It might be used for the prevention and treatment of various inflammatory diseases. However, further *in vivo* studies are needed to confirm those *in vitro* findings.

Acknowledgments

This study was supported by Inserm and the French Ministry of Education and Research. Miao Liu was granted from China Scholarship Council.

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Figure Legends

Figure 1: Effect of PDX on PMA-induced superoxide anion production by human neutrophils.

Human neutrophils (2 x 10^6 cells/mL HBSS) were incubated in the absence or presence of different concentrations of PDX for 20 min. PMA (0.162 mM) was added, and $O2^{\circ}$ production was measured by chemiluminescence technique in the presence of 10 µM luminol. One curve is representative of four experiments (A). Total chemiluminescence in each condition was quantified and presented as mean ± SEM of 4 experiments (B). Different letters indicate significant differences between the groups at $p< 0.001$ level (one way ANOVA followed by Fisher's PLSD test).

Figure 2: Effect of PDX on fMLF-induced superoxide anion production by human neutrophils.

Human neutrophils (2 x 10^6 cells/mL HBSS) were incubated in the absence or presence of different concentrations of PDX for 20 min. fMLF (0.1 µM) was added, and $O2^{\circ}$ production was measured by chemiluminescence technique in the presence of 10 µM luminol. One curve is representative of four experiments (A). Total chemiluminescence in each condition was quantified and presented as mean ± SEM of 4 experiments (B). Different letters indicate significant differences between the groups at $p< 0.005$ level (one way ANOVA followed by Fisher's PLSD test).

Figure 3: Inhibitory effect of PDX on neutrophil release of MPO.

Human neutrophils (2 x 10^6 cells/mL HBSS) were pretreated or not (control) with 5 or 10 µM PDX for 20 min, then stimulated with fMLF (0.1 µM) for 3 min. Cells were centrifuged and MPO activity (A) was determined in the supernatants using H_2O_2 and TMB as described in Materials and Methods. Different letters indicate significant difference between groups at $p< 0.001$ level. MPO protein (B) was determined by immunoblotting with anti MPO antibody. Data are presented as mean ± SEM of 4 experiments. Different letters indicate significant differences between the groups at $p< 0.05$ level (one way ANOVA followed by Fisher's PLSD test).

Figure 4: Effects of PDX on fMLF-induced $p47^{phox}$ phosphorylation on Ser345.

Human neutrophils (2 x 10^6 cells/mL HBSS) were pretreated with 5 or 10 µM PDX for 20 minutes, then incubated with or without (control) fMLF (0.1 µM) for 20 minutes. Cells were then lysed and proteins were analyzed with SDS-PAGE and immunoblotting with anti-phospho-ser345 antibody (pSer345) or anti-p47phox antibody ($p47^{phox}$) (A). Western blots from different experiments were scanned, phosphorylated and total $p47^{phox}$ were quantified by densitometry, and the intensity of phosphorylated $p47^{phox}$ was corrected for the amount of $p47^{phox}$ (B). Data are representative of 3 independent experiments using cells from different healthy donors. Results are expressed as mean ± SD (n=3). Different letters indicate significant differences between the groups at $p< 0.05$ level. One away ANOVA followed by Fisher's PLSD test.

Figure 5: Effect of PDX on the production of PGD_2 and E_2 in human neutrophils treated or not by LPS.

Human neutrophils (2 x 10^6 cells/mL HBSS) were treated in the presence or absence of 100 ng/mL LPS for 4 hours. Cells were then pre-incubated with and without 10 µM PDX for 3 min at 37°C and incubated with 10 µM radiolabelled ARA for 10 min. Lipids were extracted after acidification, and PGD_2 and E_2, representative of prostaglandin production, were separated by TLC. Their corresponding spots were detected by dichlorofluorescein, scrapped off and the radioactivity was counted. Results are expressed in nanomoles of PGD_2 and E_2 synthesized and represent the mean ± SEM of 4 determinations. * $p< 0.05$ as compared to control.

Table 1 Effect of PDX on COX activities

	Control	PDX 0.5 µM	PDX 5 µM
COX-1	3.4 ± 0.4	1.5 ± 0.04*	0.9±0.02*
COX-2	1.6 ± 0.7	0.5 ± 0.02*	0.3±0.01*

Each result is expressed in nanomoles of PGD_2 + E_2, representatives of total prostaglandin production (see methods). The results represent the mean ± SD of 3 determinations, *$p < 0.05$ as compared to control.

Table 2 Effect of PDX on the 5-lipoxygenase in activated human neutrophils

	Control	PDX 1 µM	PDX 10 µM
LTB_4s	9.0±1.6	9.0±3.0	9.1±2.4
5-HETE	0.8±0.3	0.6±0.3	0.6±0.4

Each result is expressed in nanomoles of the sum of the different 5-lipoxygenase metabolites: LTB_4s (LTB_4 + LTB_4 isomers) and 5-HETE and represents the mean ± SD of 3 determinations.

Figure 1

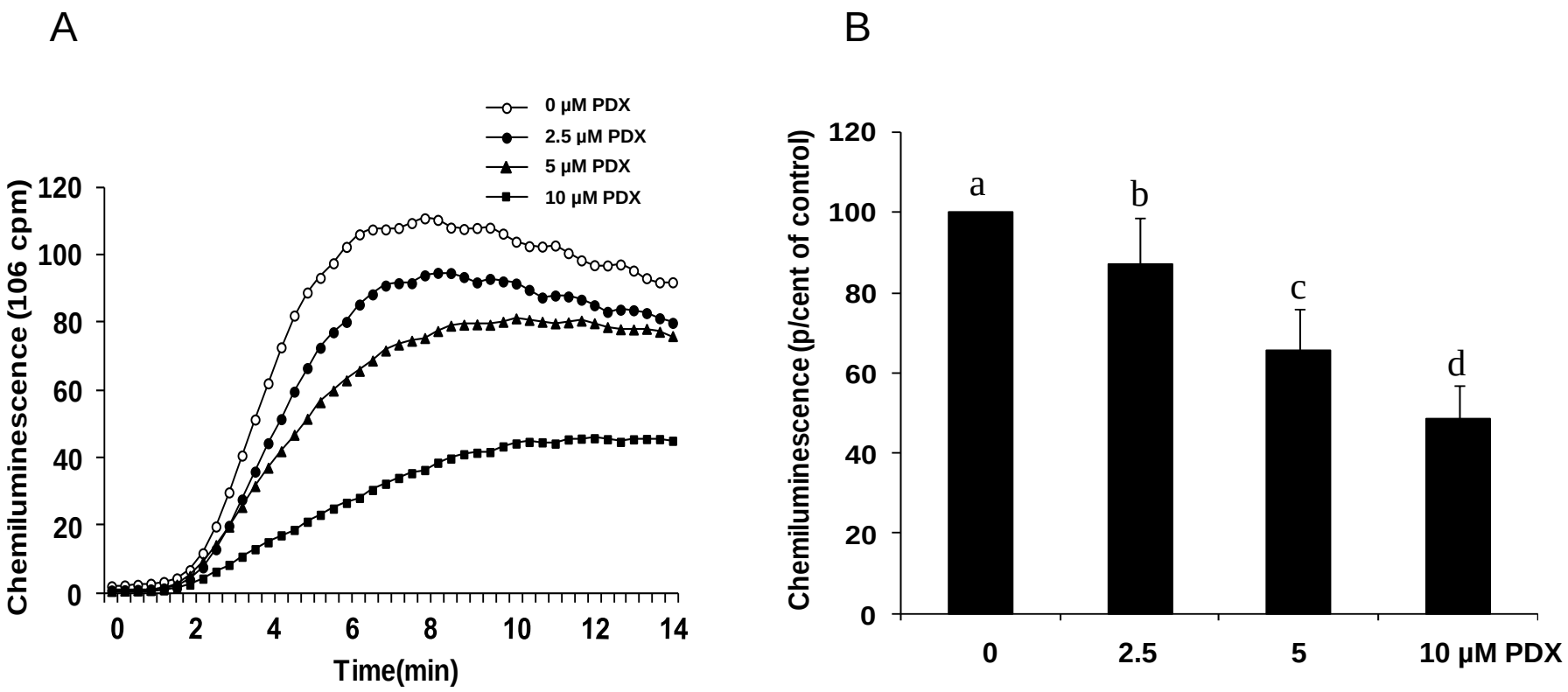

A
0 µM PDX
2.5 µM PDX
5 µM PDX
10 µM PDX
Chemiluminescence (106 cpm)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time(min)
B
Chemiluminescence (p/cent of control)
120
100
80
60
40
20
0
a
b
c
d
0
2.5
5
10 µM PDX

Figure 2

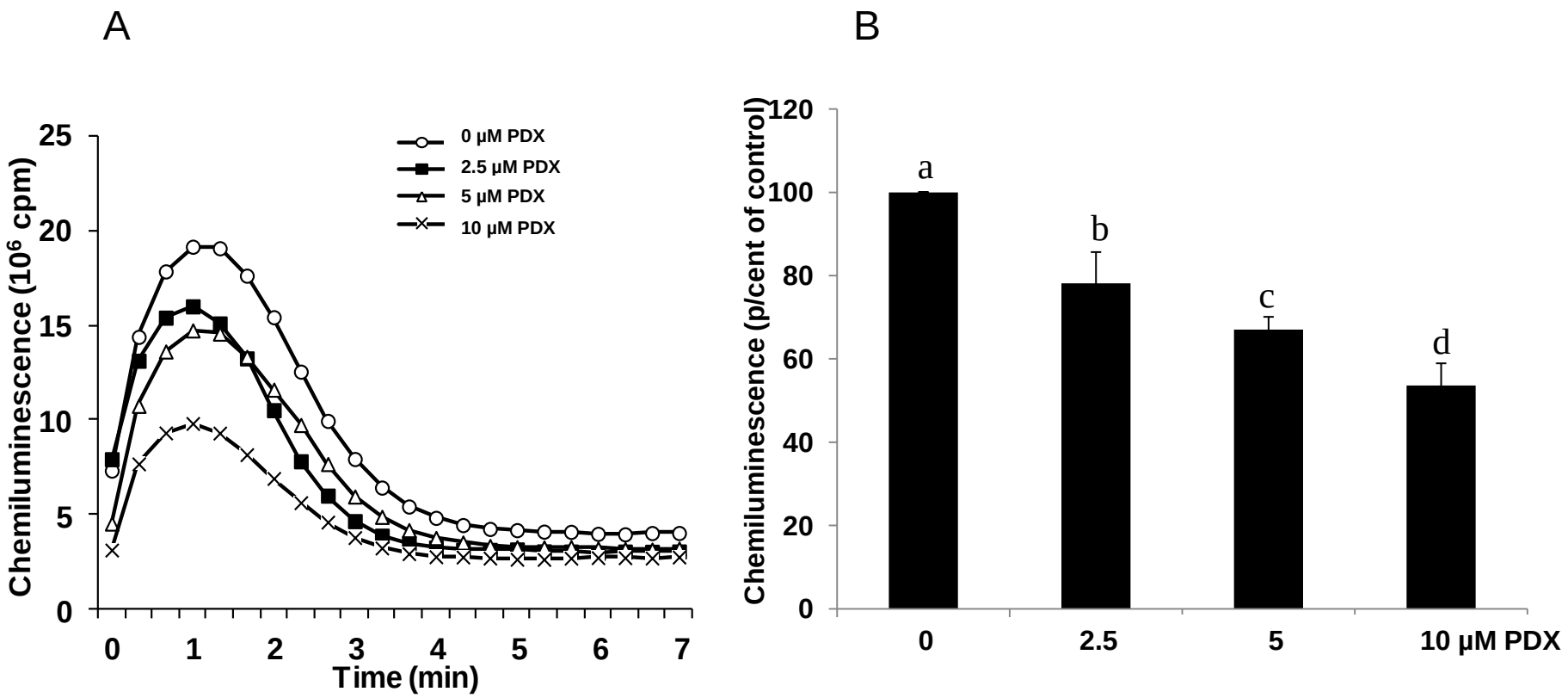

A
B
Chemiluminescence (10⁶ cpm)
Time (min)
0 µM PDX
2.5 µM PDX
5 µM PDX
10 µM PDX
Chemiluminescence (p/cent of control)
a
b
c
d
0
2.5
5
10 µM PDX

Figure 3

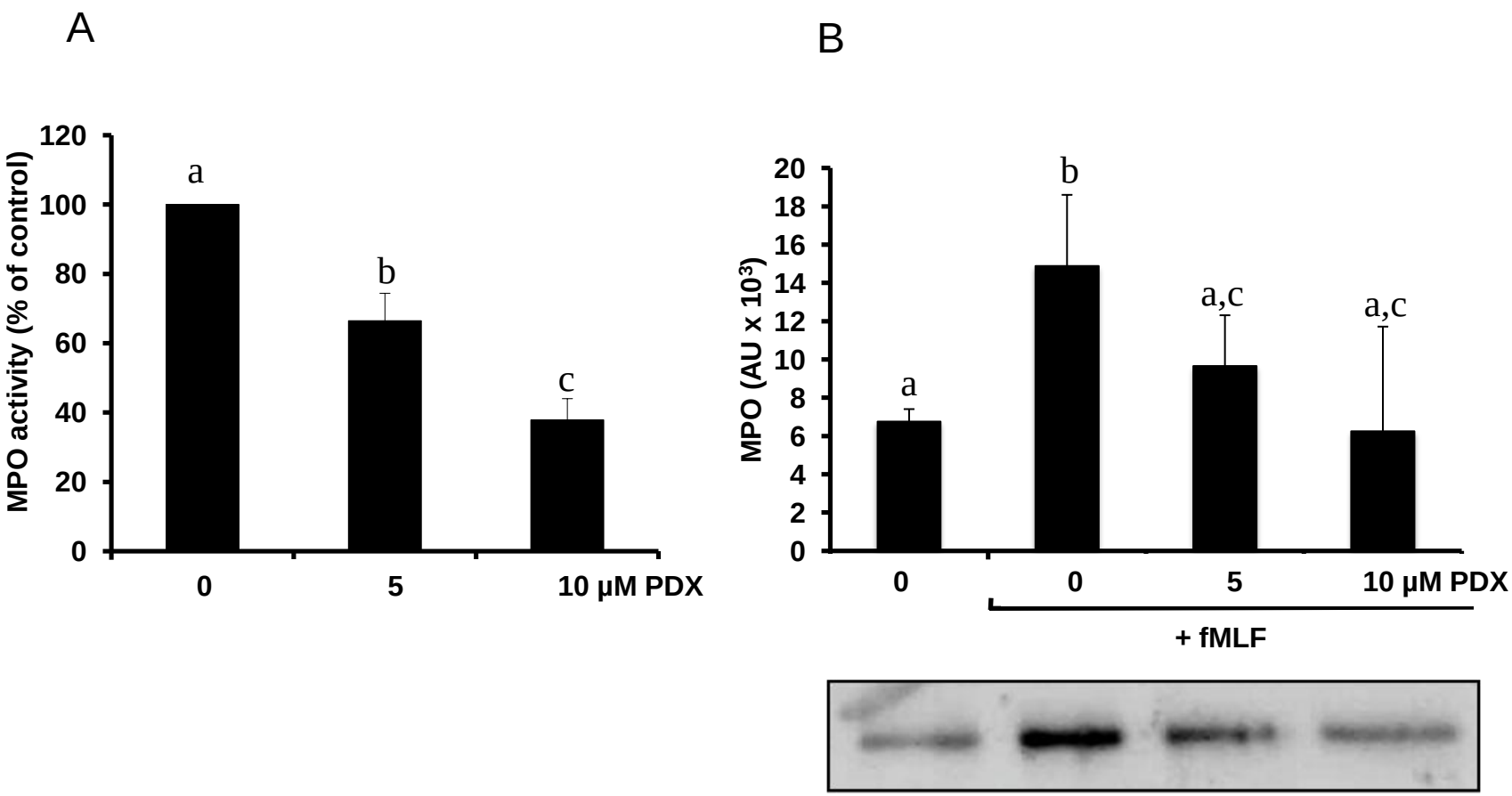
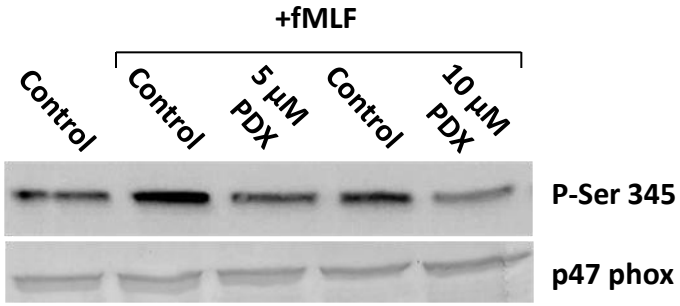

A
MPO activity (% of control)
120
100
80
60
40
20
0
a
b
c
0
5
10 μM PDX
B
MPO (AU x 10³)
20
18
16
14
12
10
8
6
4
2
0
a
b
a,c
a,c
0
0
5
10 μM PDX
+ fMLF

Figure 4

A


+fMLF
Control
Control
5 µM PDX
Control
10 µM PDX
P-Ser 345
p47 phox

B

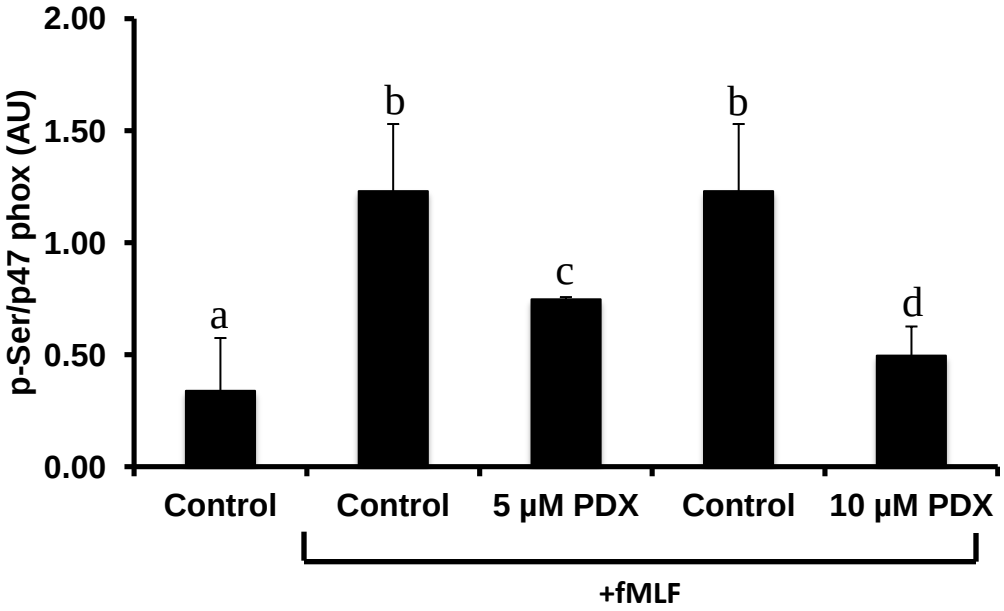
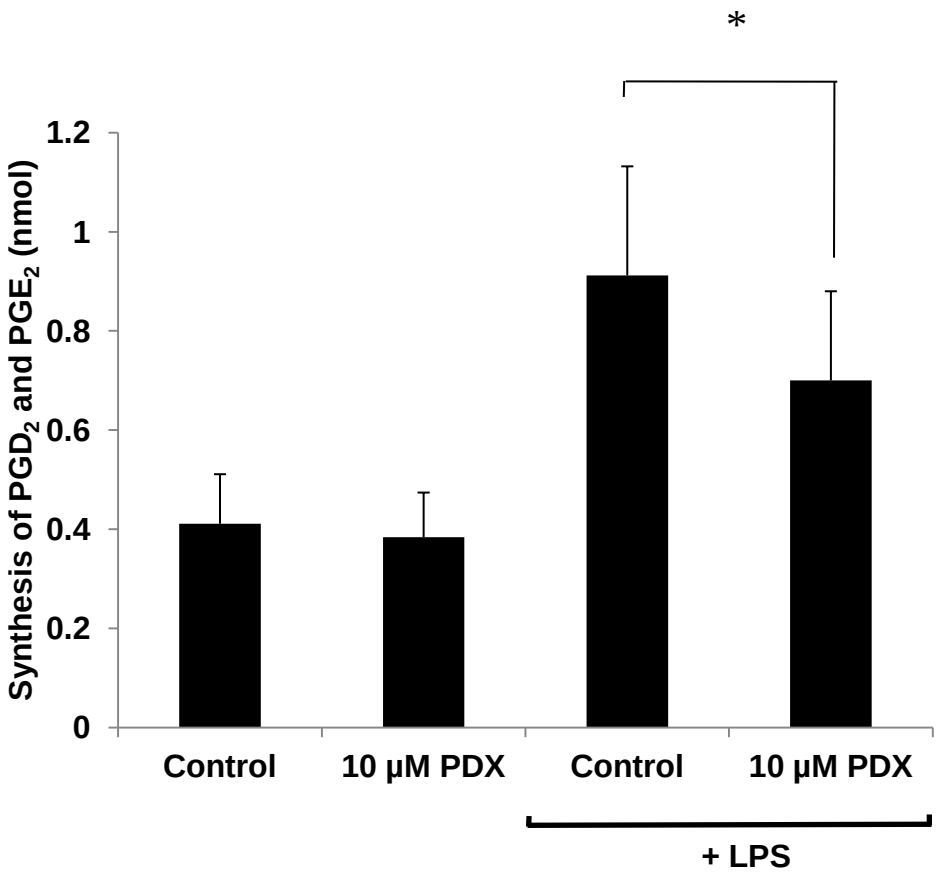

p-Ser/p47 phox (AU)
2.00
1.50
1.00
0.50
0.00
a
b
c
b
d
Control
Control
5 µM PDX
Control
10 µM PDX
+fMLF

Figure 5


*
1.2
1
0.8
0.6
0.4
0.2
0
Synthesis of PGD2 and PGE2 (nmol)
Control
10 μM PDX
Control
10 μM PDX
+ LPS